ABSTRACT

BUCHHEIT, TERESA MARIE. Effects of the Prostaglandin Analogue Misoprostol on TNF-
Alpha Release by Activated Equine Leukocytes. (Under the direction of Dr. Samuel Jones).

The magnitude of LPS-induced equine health problems cannot be over-estimated
since endotoxemia is intimately involved in the pathogenesis of gastrointestinal disorders that
cause colic, the leading cause of death in adult horses, and bacterial septicemia, the major
killer of foals less than 7 days of age. These diseases lead to huge economic losses to the
horse industry. The morbidity and mortality associated with sepsis in horses are primary
attributable to the endogenous mediators released during the host’s response to bacterial LPS.
Antimicrobials do not mitigate the effects of endotoxin and many current pharmacological
therapies, such as non-steroidal inflammatory drugs (NSAIDs), only affect part of the
inflammatory cascade, making this a difficult condition to manage effectively. The optimal
therapy likely involves methods to alter the generation of inflammatory mediators. The
capacity to neutralize TNF-α production remains a critical goal for investigating novel drugs
for use in diseases associated with systemic inflammation.

In other species, the prostaglandin analogue misoprostol has been shown to inhibit
LPS-induced TNF-α synthesis due to its ability to increase cAMP levels within leukocytes.
Given the capacity of misoprostol to inhibit TNF-α release from immune cells in other
species and its clinical use in horses for protection against NSAID-induced gastrointestinal
ulcers, the objective of the study reported here was to evaluate the effects of misoprostol on
production of TNF-α by LPS-stimulated equine leukocytes. The hypothesis of this study was
that misoprostol would suppress LPS-induced secretion of TNF-α by equine peripheral blood leukocytes.

The results presented in this study show that addition of LPS to equine leukocytes induced TNF-α secretion and misoprostol effectively blunted this response. Thus, misoprostol may be useful as an immunomodulator of inflammatory cytokine production in SIRS/sepsis. Further *in vivo* investigation is warranted.
Effects of the Prostaglandin Analogue Misoprostol on TNF-alpha Release by Activated Equine Leukocytes

by
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DEDICATION

To my parents for believing in me and supporting me to accomplish my dreams. To my husband, for standing by me. And to my canine companions, who brighten my day with their unconditional love.
BIOGRAPHY

Teresa Marie Buchheit received her Bachelor of Science and also her Doctorate of Veterinary Medicine at Purdue University. Teresa completed a one year internship in Equine Medicine and Surgery at Mississippi State University and then completed a three year Large Animal Internal Medicine Residency at the University of Tennessee College of Veterinary Medicine. She began work as a large animal veterinarian at a private practice in Louisville, TN. After a year in private practice, she returned to academia to pursue a graduate degree.
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CHAPTER 1
Literature Review

A. Equine SIRS/Sepsis

1. Introduction to Sepsis

Sepsis represents the host’s systemic inflammatory response to infection. It causes substantial human morbidity and has been ranked the 11th leading cause of death in the United States.[1] In the equine industry, sepsis remains one of the most challenging problems faced by veterinarians and the mortality rate remains quite high.[2]

The word *sepsis* has been around since the ancient Greeks meaning decay or decomposition.[3] The word was first mentioned in the poems of Homer over 2,700 years ago, where sepsis is used as a derivative of the verb “sepo” which means “I rot”.[4] The term is also found in the writings of the physician and philosopher Hippocrates (ca. 460-377 BC) who introduced the term “wound putrefaction”.[5] In the 18th century, the French chemist and microbiologist Louis Pasteur first linked microorganisms and human disease when he identified the *Streptococcus* bacteria as the etiology of puerperal sepsis.[5, 6] In 1914, Hugo Schottmüller paved the way for a modern definition of sepsis describing a source of an infection as a fundamental component of the disease: "Sepsis is present if a focus has developed from which pathogenic bacteria, constantly or periodically, invade the bloodstream in such a way that this causes subjective and objective symptoms".[5, 7]

Decades later, the focus of sepsis research shifted from the infectious agent to the host
immune response.[5] Finally the concept entered into clinical practice after Roger Bone and colleagues proposed the term “sepsis syndrome” in their landmark paper published in 1989.[5, 8, 9]

In 1991, a consensus conference of the American College of Chest Physicians and the Society of Critical Care Medicine introduced the idea that sepsis is the host’s inflammatory response to infection. However, the label sepsis is often inappropriately used to describe clinical signs (fever, tachycardia, tachypnea, and abnormal white blood cell count) in patients that may or may not have an underlying infection causing clinical parameters. In an effort to standardize nomenclature, the term “systemic inflammatory response syndrome” (SIRS) was coined to denote a clinical syndrome whose differential diagnoses included infectious as well as non-infectious causes and sepsis was reserved for SIRS due to suspected or confirmed infection.[3, 10-13] A second sepsis definition conference was held in 2001 that concluded that the 1991 definition of sepsis was still useful and further defined specific categories of sepsis.[10, 11, 14, 15]

The aim of this section of the literature review is to detail the currently used definitions of SIRS and sepsis syndromes and to present an overview of the current understanding of the pathophysiology which underline these conditions in horses. This brief review focuses predominately on the first line of defense against invading pathogens, the innate immune response, and aberrant inflammatory mediator production.

2. Definitions

The definition of sepsis and SIRS has caused much controversy and debate in the recent decades in human literature, reflecting both the complexity of the condition and the
similarity of the inflammatory response secondary to a variety of insults including both infectious and noninfectious etiologies.[13] In the equine literature, the definition of sepsis has also been the subject of some debate. One may see equine sepsis defined at the occurrence of “endotoxemia” with documented systemic bacterial infection.[16] Endotoxemia, by definition, refers to the presence of bacterial endotoxins from Gram-negative microorganisms in circulation.[17] It is well known, however, that sepsis, can also be induced by Gram-positive organisms.[16] Borrowing terms from the human literature, the following definitions are used in the horse to describe patients with sepsis syndromes:[2, 8, 17-21]

*Infection:* a pathologic process caused by the invasion of normally sterile tissue, fluid or body cavity by pathogenic or potentially pathogenic microorganisms.

*Systemic inflammatory response syndrome* (SIRS): the systemic inflammatory response to a variety of severe clinical insults manifested by two or more of the following conditions: (1) fever or hypothermia; (2) tachycardia; (3) tachypnea or hypocapnia; (4) leukopenia, leukocytosis, or increased circulating immature neutrophils.

*Sepsis:* SIRS induced by infection (bacterial, viral, protozoal or fungal).
Severe sepsis: sepsis complicated by at least one organ dysfunction; usually acute circulatory failure characterized by hypotension/hypoperfusion unexplained by other causes and readily corrected by volume resuscitation.

Septic shock: severe sepsis plus acute circulatory failure characterized by hypotension unexplained by other causes and despite adequate fluid resuscitation in association with hypoperfusion.

Multiple organ dysfunction syndrome (MODS): presence of organ dysfunction in an acutely ill patient such that homeostasis cannot be maintained. MODS is a progressive syndrome, often resulting in death.

Compensatory anti-inflammatory response syndrome (CARS): describes a patient with increased circulating anti-inflammatory mediators, leukocyte anergy, or increased susceptibility to infection.

Mixed anti-inflammatory response syndrome (MARS): describes a patient presenting with features of CARS and SIRS.
Other terminology in veterinary literature:

*Septicemia:* disease resulting from the presence of microorganisms and/or their toxins in the bloodstream.[2, 22] The term is no longer used in human literature, but is used in reference to equine neonates.

*Bacteremia:* presence of viable bacteria in the bloodstream.[2]

*Endotoxemia:* the presence of bacterial endotoxins from Gram-negative bacteria in circulation.[17]

### 3. Epidemiology

Many horses with colic and neonatal critically ill foals exhibit clinical signs similar to those described for SIRS (see discussion below). Gastrointestinal diseases (i.e., colic) remain the leading cause of death in adult horses and mortality is related to the degree of SIRS.[23, 24] Neonatal septicemia is the major killer of foals less than 7 days of age.[24] Epidemiological data pertaining to the morbidity and mortality associated with SIRS or sepsis in horses appears to be lacking.[20] A recent review of sepsis reports nearly a 50% case fatality rate associated with sepsis in a variety of veterinary species.[25] In a study of critically ill foals presenting to a teaching hospital for evaluation and treatment, Corely and colleagues reported that more than 40% of the foals evaluated had a diagnosis of SIRS.[26] In another study of adult horses presenting for evaluation and treatment of colic at a different institution, Epstein and colleagues determined that nearly 30% had evidence of SIRS.[27]
4. **Overview of Clinical Sepsis in the Horse**

Clinically, human patients are said to have sepsis if they have a documented or suspected infection in addition to at least two of the following criteria for SIRS: fever or hypothermia, tachycardia, tachypnea or hypocapnia, leukocytosis, leukopenia, or an increased number of circulating immature (band) neutrophils.[2, 20, 28] No consensus criteria for SIRS or sepsis has been established for equine patients; although, to the author’s knowledge, the topic of a consensus statement has been recently discussed by members of the American College of Veterinary Internal Medicine (unpublished). The physiologic changes typically associated with SIRS/sepsis are alterations in heart and respiratory rates, mucous membrane color, and capillary refill time.[17, 20] In severe cases, weak peripheral pulse quality, cool extremities, and poor jugular filling may be noted if the circulatory system is compromised.[20]

The following criteria for a diagnosis of SIRS in horses has been proposed (at least 2 are required):[17]

- Hyperthermia (rectal temperature above 101.5°F) or hypothermia (below 98°F; normal 98.5-101.5°F)
- Tachycardia (heart rate above 60 beats per minute; normal 30-40 beats/min)
- Tachypnea (abnormally rapid breathing) or hypocapnia
- Abnormal leukocyte count (leukopenia, leukocytosis, or >10 % band neutrophils)
- Evidence of sepsis in foals

The clinical conditions seen in horses that have been associated with the presence of SIRS include inflammatory intestinal diseases (proximal enteritis, colitis), strangulating obstructions of the gastrointestinal tract, carbohydrate overload, severe bacterial pneumonia
and pleuropnuemonia, recurrent airway obstruction, inflammatory airway disease, clostridial myositis, septic endometritis, and exertion.[20, 29-33] Besides these infectious causes, SIRS in horses, as in humans, can occur without evidence of infection or sepsis.[34] Noninfectious causes of SIRS include trauma, ischemia or restricted blood supply to tissues, immune-mediated diseases, surgery, hypo- or hyperthermia, and profound hypoxia or hemorrhagic shock.[2, 3] In neonatal foals, SIRS has been associated with bacteremia, infectious enteritis, pyelonephritis, pneumonia, septic arthritis, and perinatal asphyxia syndrome.[2, 20] 

*Rhodococcus equi* pneumonia in foals is an example of a Gram-positive organism causing signs consistent with the presence of SIRS.[35]

Sepsis is said to be severe if accompanied by MODS. In equine patients, organ dysfunction may include laminitis, evidence of coagulopathy, pulmonary dysfunction, gastrointestinal dysfunction, renal dysfunction or cardiovascular dysfunction.[20] Horses are unique in that the cascade of inflammation that occurs in SIRS frequently leads to laminar failure in the hoof.[31, 36-38] SIRS-associated laminitis without infection or sepsis may occur, as is evident by both the black walnut exposure and carbohydrate overload models of laminitis used in research settings.[31, 38, 39]

*SIRS or Endotoxemia?*

The term *endotoxemia* is frequently used in equine veterinary literature and is sometimes used interchangeably with sepsis and SIRS.[17] The term SIRS may be more appropriate to describe the above clinical status of horses because endotoxemia actually refers to the presence of endotoxin from Gram-negative bacteria in circulation and endotoxin is rarely measured in the blood in clinical cases.[17, 40] In addition, recent investigations indicate
that the clinical signs associated with inflammatory diseases ultimately are the result of inflammatory mediators synthesized by the host’s cells rather than the presence of endotoxins in circulation.[17] This is further supported by the findings that SIRS develops in Gram-positive infections, as well as non-infectious insults.[25, 41]

The impact of endotoxin causing disease in horses, however, cannot be overlooked. Horses, as a species, are particularly sensitive to endotoxin and it is likely a common cause of SIRS in adult horses.[42] As mentioned above, colic is the leading cause of death in horses and mortality is closely related to the degree of SIRS.[43] The lumen of the equine large colon contains a large reservoir of bacteria and endotoxin, and when the integrity of the mucosal barrier is compromised, as occurs with inflammation or ischemia of the intestinal wall, endotoxins can gain access to the circulation, inciting a systemic inflammatory response.[31, 44] In addition, many of the above-mentioned diseases associated with the presence of SIRS are caused by Gram-negative bacteria.[31] In a recent study measuring endotoxin in the plasma of adult horses, approximately 30% of the study population that presented to the hospital with intestinal disease had detectable levels of endotoxin.[40] This finding was similar to some other studies.[45-47]

Treatment

The switch in terminology from endotoxemia to sepsis and SIRS has influenced how veterinarians medically view and treat horses with SIRS. Treatment focus has turned to decreasing inflammation. Methods to alter the generation of inflammatory mediators have been recently investigated or are currently being investigated.[48-55] Current therapeutic
interventions for these syndromes in horses have been well-described and are beyond the scope of this review.[20, 21, 31, 34, 43, 56]

5. **Overview of the Pathophysiology of Sepsis and SIRS**

5.1 **Inflammation during sepsis/SIRS**

The similarity of the inflammatory response, despite the variety of underlying etiologies, is suggestive of a common pathophysiology of SIRS and sepsis. Although infection may be responsible for initiation of an inflammatory response, the inflammatory process itself results solely from the production of endogenous mediators.

5.1.1 **Stimulation of the innate immune response**

Host defense against severe infection is primarily the function of innate immunity since the adaptive immune system, although ultimately required to clear an insult, takes days to respond.[17, 20, 52, 57] The innate immune response is reliant on the ability of the immune system to distinguish between self and microbial products (“non-self”).[57] Microbes contain or release a variety of products that are recognized by the host as foreign. These are components that are essential for microbial survival and are unique to microbial pathogens and not produced by the host. These are excellent targets for innate immune recognition because the microbe does not modulate or modify these components such that they would evade host detection. These highly conserved structural motifs are known as pathogen-associated molecular patterns (PAMPs).[25, 52, 57] PAMPs include various
bacterial cell wall components such as endotoxin (lipopolysaccharide [LPS]), peptidoglycan, lipoteichoic acid, and lipoprotein; as well as flagellin, bacterial DNA, and viral double-stranded RNA.[25, 41, 52] In addition to PAMPs, endogenous molecules released from necrotic or dying cells are also recognized by the innate immune system. These endogenous molecules are known as “alarmins” or danger-associated molecular patterns (DAMPs) and include intracellular proteins such as heat shock proteins as well as protein fragments from intracellular material (fibrinogen, fibronectin, hyaluronate fragments, biglycans, and high-mobility group box-1 [HMGB1]).[25] In many cases, the inflammatory response serves as a protective function and is a critical component of innate immunity, but in some cases, it contributes to the pathophysiology of SIRS and sepsis.[17, 25]

Gram-negative LPS represents a classic example of a PAMP and is one of the best studied activators of innate immunity.[58] LPS is a component of the outer membrane of Gram-negative bacterial cell walls and is released upon rapid proliferation and cell death.[24, 31, 59, 60] LPS is composed of 3 distinct portions: a hydrophilic O-specific side chain, core polysaccharide region, and a hydrophobic lipid A moiety.[17, 58] This lipid A moiety is the bioactive component of LPS that is associated with most of the toxic effects and is highly conserved among Gram-negative bacteria.[31, 57] It anchors the LPS molecule in the bacterial cell membrane and is not available for interaction with inflammatory cells until released.

Upon encountering immune cells, LPS elicits a robust inflammatory response. Macrophages and circulating monocytes, for example, respond to LPS by producing a number of effector molecules. Perhaps the best studied of these is tumor necrosis factor-
alpha (TNF-α). TNF-α, in turn, induces the production of other inflammatory mediators, such as interleukin-1 beta (IL-1β) and IL-6, and acts on other inflammatory cells to increase expression of adhesion molecules and produce antimicrobial mediators such as nitric oxide and reactive oxygen species. Collectively, this leads to an acute inflammatory response that can curb pathogen spread and alert the more specialized adaptive immune system to the presence of infection. TNF is a very important molecule, but its presence needs to be strictly regulated. It has enormous potential not only to cause acute inflammation important in host defense, but also can drive the pathological inflammatory responses that occur in SIRS and sepsis.

5.1.2 Overview of how LPS is recognized

LPS molecules tend to aggregate and form micelles in plasma based on their amphipathic nature. From work over the last 20 years, it is known that LPS binding protein (LBP), an acute phase protein, binds circulating LPS and enhances its activity by removing individual LPS molecules from micellar aggregates in plasma. LBP transfers LPS onto a receptor called cluster of differentiation antigen 14 (CD14), which can exist either as a soluble molecule in plasma or a membrane-bound form on the surface of inflammatory cell types including mononuclear phagocytes, neutrophils and endothelial cells. CD14, in turn, transfers LPS to a signaling receptor complex since CD14 itself lacks a transmembrane domain and does not have signaling potential (see later discussion on signaling). Upon encountering LPS, a multitude of intracellular signaling pathways are activated leading to activation of NFκB, MAP kinases, PI3 kinase, and transcription...
factors that traffic to the nucleus to turn on expression of a vast array of inflammatory genes, such as TNF-α and related cytokines, that are responsible for the clinical manifestations of sepsis.[25, 62]

5.1.3 LPS/TLR signaling

Key insights into LPS signaling involved the discovery of the Toll gene in *Drosophila.*[20, 25, 31, 57] Toll (a pattern recognition receptor [PRR]) exists in all mammals and plays a critical role in the early innate immune response by sensing microorganisms (PAMPs) and is involved in sensing endogenous danger signals (DAMPs).[63] In addition to the Toll-like receptors (TLRs), other families of PRR’s include the nucleotide-binding leucine-rich repeat containing receptors (NLRs; previously designated as the nucleotide-binding oligomerization domain [NOD]-like receptors), and the retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs).[25] Of these receptors, only the TLRs have been investigated in equine cells[17]; therefore, the NLR and RLR families of PRRs are not considered further in this review. The structure and function of these receptors’ families were recently reviewed in detail.[64] There are many Toll-like receptors and 10 have been discovered in the horse; a number similar to that identified in humans.[25, 57, 63] TLR2 is essential for recognition of a variety of PAMPs including lipotechoic acid (from Gram-positive bacteria), peptidoglycan (from Gram-positive and Gram-negative bacteria), polysaccharides, and lipoproteins.[25] TLR4, the prototypical member of the TLR family, is predominately activated by LPS and is the focus of this review. Increased expression of the TLR4 gene has been reported in both foals and adult horses with SIRS/sepsis.[25, 65]
The Toll-like receptors are related to the IL-1 receptor family.[31, 57, 62] These TLRs are type I transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats, a transmembrane domain, and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 (TIR) domain.[25, 31, 57] The leucine-rich repeats are thought to be involved in ligand interactions (recognizing specific PAMPs) and the intracellular TIR domain is the active segment of the TLR molecule and facilitates intracellular signaling involved in signal transduction.[31, 57] The extracellular portion of TLR4 interacts with an accessory protein, myeloid-differentiation factor-2 (MD-2), on the cell surface.[31, 57, 59] TLR4, in association with MD-2, represents the actual signaling portion of the LPS receptor complex.

Upon ligand binding, TLRs dimerize or associate with other receptors.[25, 57] The TIR domain interacts with other TIR-domain-containing intracellular proteins. Four intracellular adaptor proteins that contain a TIR domain have been described: myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP, also known as MyD88-adapter-like [Mal]), TIR-domain-containing adaptor protein-inducing interferon gamma (TRIF, also known as TIR-domain-containing adaptor molecule 1 [TICAM1]), and TRIF-related adaptor molecule (TRAM, also known as TIR-domain-containing adaptor molecule 2 [TICAM2]).[25, 62] MyD88 and TRIF define two independent signaling pathways, while TIRAP and TRAM act as bridge molecules.[62]

TLR signaling consists of at least two distinct pathways: a MyD88-dependent pathway, which leads to production of inflammatory cytokines, and a MyD88-independent pathway (TRIF-dependent) associated with induction of Type I interferons and interferon-
inducible genes.[25, 58] Since the focus of this review is mainly on inflammatory mediators and their role in sepsis, the MyD88-dependent pathway is reviewed in more detail below. An overview of the MyD88-independent (TRIF) pathway is discussed below as it relates to the expression of the anti-inflammatory mediator IL-10, but a more detailed review can be found by Lu et al.[58]

All of the TLRs, except TLR3, can signal through MyD88.[25, 61, 62] Upon activation by PAMPs or DAMPs, TLRs hetero- or homodimerize, inducing recruitment of adaptor proteins via the cytoplasmic TIR domain.[62] Individual TLRs induce different signaling responses by usage of different combinations of adaptor molecules.[58] For example, TLR2 and TLR4 signaling require TIRAP to recruit the MyD88 adaptor protein.[17] Recruitment of MyD88 has two effects that occur in the cytosol: (1) activation of a chain of kinases in the IL-1 receptor-associated kinase (IRAK) family, ultimately leading to degradation of IκB (inhibitor of κ light chain gene enhancer in B cells) proteins and subsequent activation of the transcription factor nuclear factor κB (NF-κB) and (2) phosphorylation of the mitogen activated protein (MAP) kinases and activation of additional transcription factors (activator protein-1 [AP-1]).[17, 31] More specifically, MyD88 recruits IRAK1 and IRAK4. IRAK4 subsequently activates IRAK1 by phosphorylation and IRAK1 and IRAK4 dissociate from the MyD88-TLR complex. Next, IRAK1 and IRAK4 interact with TRAF6 (TNF receptor-associated factor 6), which in turn activates the NFκB pathway; ultimately stimulating the transcription of pro-inflammatory cytokines (including TNF-α, IL-1β, IL-6 and the chemokine IL-8) that direct the adaptive host response.[58, 62] Activation of the downstream MAP kinase pathways also has a role in the expression of pro-
inflammatory cytokines.[58] The most influential inflammatory cytokines involved in SIRS are described in more detail later in this review.

In species studied to date, LPS signaling through TLR4 also utilizes the TRIF-dependent pathway.[17, 61, 62] In contrast to rapid activation of NFκB by the MyD88-dependent pathway, the response is delayed when the TRIF-dependent pathway is used.[61, 62] Activation of TRIF by LPS leads to rapid production of TNF, which then acts in an autocrine manner to activate NFκB. This delayed activation of NFκB may be due to the time required for TNF synthesis.[62] In addition to delayed activation of NFκB, the TRIF-dependent pathway also activates interferon (IFN)-regulator factor (IRF3) and leads to stimulation of interferon-beta (IFN-β) and subsequent induction of IFN-responsive genes (CCL5 [RANTES; regulated on activation, normal T-cell expressed and secreted] and IP-10 [interferon gamma-induced protein 10]).[17, 61] The interferons (IFNs) are implicated in anti-viral defense and will not be further discussed in this thesis. In a recent review of SIRS in horses with colic, Moore includes a discussion on the importance of TLRs in the recognition of viruses.[17] Activation of NFκB via the TRIF-dependent pathway results in increased expression of IL-10, an anti-inflammatory mediator.[17] Stimulation of the TRIF-dependent pathway also leads to maturation of monocyte-derived dendritic cells, an important link between the innate and adaptive immune responses.[61] Interestingly, Figueiredo et al. found that in contrast to the LPS-induced responses in other species, LPS activation of TLR4 (as well as TLR2) in equine monocytes occurs almost exclusively through the MyD88-dependent pathway.[61] The authors found that expression of the TNF-α, IL-1β, and IL-6 genes (hallmarks of MyD88-dependent activation) were induced by LPS.
in equine peripheral blood monocytes. In contrast, even high concentrations of LPS failed to induce expression of genes associated with the TRIF-dependent pathway.[61] Activation of TLR2 or TLR4 in equine monocytes leads to expression of the anti-inflammatory mediator IL-10; however, the magnitude of IL-10 expression was found to be far greater for activation of TLR3, which was the only TLR found to utilize the TRIF-dependent pathway in horses. This is particularly important because the TRIF-dependent pathway is viewed as being anti-inflammatory in nature, whereas the MyD88 pathway promotes a pro-inflammatory state. In other species, switching occurs between the MyD88-dependent and TRIF-dependent TLR4 signaling pathways, creating a balance between pro-inflammatory and anti-inflammatory mediators, which determines the fate of the host innate immune response.[17] In equine monocytes, it appears this switching between these signaling pathways is absent.[17] Given the horse’s sensitivity to LPS, the fact that TLR4 in equine monocytes only signals through MyD88, and the lack of induction of TRIF-dependent anti-inflammatory cytokines (such as IL-10) by equine TLR4, the pro-inflammatory responses of LPS in horses may be far stronger than those occurring in other species with the innate immune response frequently culminating in SIRS.[17] (See discussion of SIRS, MARS, and CARS later in this review).

5.2 Role of Cytokines in Sepsis

Cytokines are soluble, low molecular weight glycoproteins which act as inflammatory mediators orchestrating the immune and inflammatory responses.[52] Individual cytokines are produced by multiple cell types and act on multiple target cells in different ways. At low concentrations, cytokines have a paracrine or autocrine effect, whereas at increased
concentrations, such as in SIRS and sepsis, the cytokines have endocrine effects and act systemically.[66]

Several cytokines have been implicated in the development of SIRS and sepsis, including TNF-α, interleukin (IL)-1 β, IL-8, IL-6, IL-10, IL-4, IL-13, interferon-gamma (IFN-γ), and transforming growth factor-beta (TGF-β).[52] Circulating concentrations of several cytokines have been shown to be linked to morbidity and mortality in sepsis.[25, 40, 67, 68] Increased serum concentrations of TNF-α have been found to correlate with mortality in equine acute abdominal disease and in septic foals.[69-71] The production of cytokines in sepsis (“cytokine storm”) has previously been referred to as a cascade.[52, 72] This, however, is misleading, suggesting an amplification pathway. More appropriately, the interaction of cytokines should be considered instead as a network.[52] In this review, specific attention is directed toward three of the most influential pro-inflammatory mediators operating in SIRS and sepsis (TNF-α, IL-1, and IL-6) because their release has been the most closely associated with morbidity and death.[12, 52]

As a counterbalancing force to the secretion of pro-inflammatory cytokines, several anti-inflammatory mediators are produced (see discussion later in this review). These mediators suppress the synthesis and action of pro-inflammatory mediators and their detailed review, with the exception of IL-10, is not reviewed here. A recent review by Schulte et al. will provide the interested reader with a more in-depth detailed discussion of the anti-inflammatory cytokines involved in sepsis.[52]
5.2.1 TNF-alpha

TNF, also known as cachexin, was identified in 1975 as a macrophage-derived factor that could “necrotize” tumors in mice.[66, 73, 74] TNF-α is a classic pro-inflammatory cytokine produced predominately by activated macrophages and T cells, but also by a number of other immune cells, including mast cells, B cells, natural killer (NK) cells, neutrophils, and endothelial cells.[25] TNF-α and IL-1β are the prototypic inflammatory cytokines that mediate many of the immunopathological features of SIRS and sepsis.[52] TNF-α has been established as an early regulator of the immune response, with its release from macrophages beginning within 30 minutes after an inciting event.[52]

TNF-α and IL-1β affect the hypothalamic set-point producing fever, as well as increase the synthesis of each other and induce the transcription of genes of other cytokines (IL-6, IL-8, and IL-10), lipid mediators (phospholipase A₂, cyclo-oxygenase [COX]-2) and inducible nitric oxide synthase (iNOS).[20, 25, 52] This leads to the production of several other important mediators such as platelet-activating factor (PAF), prostaglandin E₂, leukotriences, and nitric oxide.[20] Because of this unique ability to orchestrate an array of downstream cytokine cascades, TNF-α is considered to be a “master regulator” of inflammatory cytokine production.[52] A review of the molecular mechanisms behind TNF’s actions has been recently published.[74] TNF-α enhances production, activation and differentiation of macrophages as well as prolonging their survival.[52] Many of the classic features of inflammation can be attributed to the actions of TNF-α upon the endothelium. TNF-α induced production of nitric oxide and COX-2 leads to vasodilation and vascular stasis.[25] TNF-α also has a role in leukocyte activation, adhesion to the endothelium, and
extravasation to tissues. TNF-α up-regulates the expression of endothelial cell adhesion molecules (E-selectin, intracellular adhesion molecule [ICAM]-1, and vascular cell adhesion molecule [VCAM]-1) that lead to tethering of leukocytes to the endothelial wall and their transmigration through the vascular endothelium into tissues.[25, 52] In addition to up-regulation of iNOS, COX-2, and adhesion molecules; TNF-α also is involved in activation of the coagulation cascade in sepsis leading to the dysregulation of coagulation.[12, 25, 52] Specifically, TNF-α induces the expression of the procoagulant protein tissue factor (TF) and down-regulates the anticoagulant protein thrombomodulin.[25] TNF-α is also known to induce type I acute phase proteins involved in inflammation.[12, 25] In addition, TNF-α has been implicated in a number of studies as an important mediator of myocardial dysfunction.[66, 72, 75, 76]

TNF-α is the earliest cytokine to appear in circulation in studies of experimental endotoxemia in horses.[77] Figueiredo et al. showed that the induction of TNF-α gene expression after stimulation of equine monocytes is rapid (1 hour).[61] Increased circulating TNF-α has been detected in some clinical cases of colic and a marked increase in TNF-α levels have been associated with higher mortality.[45, 78-80] The importance of TNF-α in the pathogenesis of sepsis cannot be underestimated since the sole administration of TNF-α has been shown to induce the pathophysiological events associated with SIRS in experimental study animals.[81]
5.2.2 IL-1beta

IL-1, also known as catabolin, was first discovered in 1972 and originally named lymphocyte-activating factor, but it wasn’t until 1985 when the distinct molecule IL-1β was discovered. [82] IL-1 is released by immune cells in response to, or in parallel with, TNF-α. [12] As already mentioned, IL-1β acts synergistically with TNF-α in acute inflammation. [25, 52, 83] IL-1β acts to induce the synthesis of adhesion molecules and cytokines by endothelial cells, encouraging leukocyte activation, endothelial tethering, and migration into the tissues. [12, 25] IL-1 also up-regulates iNOS and COX-2 production, is involved in inflammation-induced activation of coagulation, and also acts as a major endogenous pyrogen producing fever. [25, 52] IL-1 induces production of type I acute phase proteins by the liver. [25]

Increased IL-1β has been measured in the serum of horses within 3-4 h after LPS administration. [67] Figueiredo et al. found that the induction of IL-1β gene expression in stimulated equine monocytes is sustained for a much longer time period (up to 20 h) after stimulation than TNF-α. [61] As seen with TNF-α, the clinical picture of septic shock can be reproduced by the administration of IL-1 by itself in experimental study animals, further providing evidence of the importance of these two cytokines in the pathogenesis of sepsis. [12, 83, 84]

5.2.3 IL-6

IL-6 is another important pro-inflammatory cytokine that is increased in sepsis. [20, 25, 52] It is produced primarily by macrophages and T cells after exposure to TNF-α and IL-
1β.[12, 85] A key function of IL-6 is mediation of the acute phase response. It is integral for fever response and is a potent stimulator of the production of Type II acute phase proteins by the liver.[25, 52] Another major action of IL-6 includes stimulating the final differentiation of B cells into antibody producing cells (an integral component of the adaptive immune response).[12, 20, 25, 86] IL-6 also effects T cell proliferation and differentiation, and NK cell activities.[16] In addition, IL-6 appears to have some immunomodulating activities by suppressing the production of TNF-α and IL-1β.[12, 25, 87] IL-6 may be considered a prognostic indicator, and, like TNF-α and IL-1, high concentrations of IL-6 have been correlated with a poor outcome in sepsis.[20, 25, 65, 85] In contrast to TNF-α and IL-1β, administration of IL-6 by itself to animals or humans does not induce a sepsis-like state.[52, 83] IL-6 may also be involved with myocardial dysfunction in septic shock.[52]

**Cytokine kinetics: Specific studies in horses**

A multitude of studies in horses have shown increased TNF-α, IL-1β, and IL-6 in horses in SIRS conditions.[41, 45, 61, 67, 68, 86, 88-90] The timing and magnitude of cellular responses is likely important to the establishment of SIRS. For example, as already mentioned, Figueiredo et al. found that in activated equine monocytes, the induction of TNF-α, IL-1β, and IL-6 gene expression occurs at different times after stimulation and lasts for different lengths of time. The induction of TNF-α gene expression was rapid and transient (1 hour post-stimulation), whereas induction of IL-1β gene expression was sustained for a much longer time interval (for up to 20 hours). IL-6 gene expression was delayed and sustained (from 4 to 20 h).[61] In studies measuring cytokine levels in the blood of horses after LPS
administration, the mean peak serum activity of TNF-α was observed between 1-2 h, and IL-1β and IL-6 peaked between 3 and 4 h.\cite{67, 86, 91, 92} Nieto et al. investigated gene expression of cytokines measured in the blood of horses after LPS administration. An increase in TNF-α gene expression was observed from 30 min to 2 h with a peak at 60 min. Expression returned to baseline values by 3 h post-LPS infusion. Cytokine gene expression for IL-1β appeared in circulation within 30 min and peaked at 60 min post-LPS infusion. Expression of IL-6 peaked at 90 min after LPS administration. In addition, IL-1β and IL-6 gene expression remained significantly higher than baseline at 3 h after LPS infusion.\cite{89}

\subsection*{5.2.4 IL-10}

Serum concentrations of IL-10, an anti-inflammatory cytokine, also are increased in SIRS and sepsis.\cite{25} IL-10 is produced by many cell types of the innate and adaptive immune systems, including macrophages, NK cells, B cells and T cells.\cite{52} The critical role of IL-10 is mediating the balance between pro-inflammatory and anti-inflammatory processes.\cite{93} This cytokine suppresses immune function and acts through several different pathways to down-regulate the production of pro-inflammatory cytokines, hence reducing inflammation and restoring homeostasis. IL-10 has been shown \textit{in vitro} to inhibit the release of TNF-α, IL-1β, and IL-6 from leukocytes.\cite{94, 95} Additionally, it also induces the production of other inhibitory cytokines, IL-1 receptor antagonist (IL-1Ra) and soluble TNF receptors (sTNFRs); thereby, neutralizing the pro-inflammatory actions of IL-1 and TNF-α.\cite{25, 52} Despite these clearly protective effects of IL-10, the actions of IL-10 may not increase survival, especially if early reversible sepsis has transitioned to irreversible septic
In clinical situations, increased IL-10 concentrations were associated with mortality in septic foals.[96]

5.3 Leukocytes, tissue injury and the inflammatory response

Leukocyte activation and recruitment is integral to the successful functioning of the innate immune system. Detailed mechanisms whereby inflammatory cells are activated in sepsis are beyond the scope of this review. Neutrophils are essential for bacterial killing; however, they can also act as a double-edge sword and are implicated in causing collateral damage to surrounding tissue as they indiscriminately release an arsenal of constituents including reactive oxygen species (ROS), antimicrobial peptides, and serine proteases (such as elastase and matrix metalloproteinases which degrade structural proteins).[12, 97, 98] For a comprehensive overview of the functions of neutrophils, the reader is referred to recent reviews highlighting the role of neutrophils in the immune system and in sepsis and organ failure.[97-100] In the inflammatory response, the interaction of leukocytes and endothelial cells plays a pivotal role. Vascular injury is a major contributing factor in the development of organ dysfunction.[31] There is an immediate increase in vascular permeability, mediated by vasoactive molecules released by damaged tissues.[31] With an appropriate stimulus, circulating neutrophils marginate to the vessel periphery, roll along the surface of the vessel wall, adhere to the endothelium (facilitated by endothelial intracellular adhesion molecules), and ultimately migrate from the vasculature to the sites of injury or inflammation.[97, 100] The molecules involved in this interaction will not be detailed in this review. In brief, “rolling” is mediated through pro-inflammatory cytokine-induced expression of selectins on
leukocytes and endothelium.[97, 100] Adhesion occurs through binding of leukocyte β₂-integrins to endothelial intracellular adhesion molecule-1 (ICAM-1).[97, 100] Adherent leukocytes are then able to diapedese through the vessel wall and migrate into the tissues. Neutrophil transmigration was recently reviewed by Voisin and Nourshargh.[101] The interaction of neutrophils with the vascular endothelium initiates a cascade of cellular interactions that result in endothelial damage, systemic hypotension, capillary leakage, and subsequent development of multiple organ failure (MOF).[31, 42, 97]

5.4 Dysregulation of coagulation during sepsis

Cytokine production due to SIRS/sepsis can also have a procoagulant effect on patients.[20, 25, 31] It is estimated that up to 30-50% of equine patients develop disseminated intravascular coagulation (DIC).[102] A detailed description of the complexities of the interaction between inflammation and coagulation is beyond the scope of this review; however, a comprehensive review of coagulopathy in SIRS and sepsis in the critically ill equine patient has recently been published.[102] One key component activating the coagulation cascade is tissue factor (TF) production from endothelial and mononuclear cells.[20, 25] This cascade results in the conversion of prothrombin to thrombin followed by generation of fibrin from fibrinogen. Breakdown of fibrin by plasmin is impaired due to high concentrations of plasminogen-activator inhibitor-1, resulting in inappropriate microthrombi formation in small vessels leading to impaired blood flow to tissues and development of multiple organ failure. DIC is a dynamic process, with consumption of platelets and coagulation factors that may paradoxically result in clinical bleeding.[20, 102]
5.5 The balancing forces: SIRS/MARS/CARS

In attempts to limit the overwhelming inflammatory response that develops in SIRS, a compensatory anti-inflammatory response known as CARS occurs at some point in sepsis.[5, 20, 25] It is debatable when the anti-inflammatory response occurs and it likely varies from patient to patient.[5, 25] Classic mediators involved in CARS include anti-inflammatory mediators that inhibit macrophage activation (IL-10, IL-4, IL-13, transforming growth factor [TGF]-β, prostaglandin E2 [PGE2], glucocorticoids), antagonists to the receptors of pro-inflammatory cytokines (IL-1 receptor antagonist [IL-1ra]), and soluble TNF receptors (TNFR1 and TNFR2).[2, 103] The production of these mediators leads to inhibition of cytokines (IL-1β, TNF-α), free radical production, macrophage cytotoxic activity, LPS-induced tissue factor expression and procoagulant activity, and vascular adhesion molecules.[2, 20] Excessive activity of the anti-inflammatory response may result in immunosuppression (sepsis-induced immunoparalysis) during or after a severe inflammatory response.[52] Additionally, MARS was coined to reflect features of both SIRS and CARS.[85] A septic patient can undergo alternating shifts toward either SIRS or CARS. Some have proposed a “Sepsis: Always in MARS” paradigm that implies a constant fluctuation of pro- and anti-inflammatory mediators in the blood over the course of sepsis vs. the traditional view that the hyperinflammatory SIRS present at the onset of sepsis was overall pro-inflammatory, and over time, the host entered an overall anti-inflammatory state.[85, 104, 105]
If an imbalance develops between CARS and SIRS, homeostasis is violated and a clinical progression towards MODS may occur.[14]

6. Conclusion

SIRS and sepsis are characterized by a full-blown, systemic activation of immune responses due to the release of high levels of PAMPs and DAMPs from invading microorganisms and/or damaged tissue, which leads to overstimulation of immune cells and a loss of control over inflammatory responses. As a result, SIRS/sepsis is accompanied by a “cytokine storm”. As written by the International Sepsis Forum, sepsis is “a life-threatening condition that arises when the body’s response to an infection injures its own tissues and organs”. [3] The SIRS response can be initiated by non-infectious insults as well. CARS, the counter-regulatory anti-inflammatory response, seeks to limit SIRS-mediated damage. Sepsis is acknowledged to be an extremely dynamic process with considerable heterogeneity in the host response. [52] Many patients are likely in a balance between SIRS and CARS that varies over time.

B. A Novel Immunomodulating Treatment Strategy for SIRS/Sepsis?

1. Overview of Prostaglandin’s (PG’s)

There are 4 principle bioactive PG’s: prostaglandin E₂ (PGE₂), protacyclin (PGI₂), prostaglandin D₂ (PGD₂), and prostaglandin F₂α (PGF₂α). Endogenous prostaglandins play a
key role in the generation of the inflammatory response and act as autocrine and paracrine
lipid mediators in the body.[106, 107]

2. PG Receptors

PG’s exert their effects by activating rhodopsin-like-7-transmembrane-spanning G-
protein-coupled receptors.[107] Multiple prostanoid receptors exist: PGE receptor (subtypes
and PGI receptor (IP).[107] The focus of this brief overview is on the PGE receptor subtypes.
The receptors for PGE are present on many cells of the immune system.[108]

The E-prostanoid receptor subtypes are coupled with different G-proteins, each
mediating unique intercellular signaling pathways that mediate the effects of receptor
activation on leukocyte function.[106]. The complexities of these intracellular signaling
pathways are beyond the scope of this review. Briefly, EP1 is coupled with the Gq-protein,
which signals through the phospholipase C pathway, activating phosphatidylinositol
metabolism ultimately increasing intracellular calcium (Ca^{2+}) concentration. EP2 and EP4
are coupled with the Gs-protein and activate adenylate cyclase, increasing cyclic adenosine
monophosphate (cAMP) levels and signaling through the protein kinase A pathway. EP3 has
multiple splice variants, the exact number differing between species, and are coupled to
different signaling systems. EP3 isoforms can couple with the Gi- or G12- proteins leading to
increased intracellular Ca^{2+} concentration, inhibition of cAMP generation, and activation of
the small G-protein Rho (G_{Rho}).[107-109] A review by Breyer et al. will provide the
interested reader with a more in-depth discussion on the roles of the individual prostanoid receptor subtypes and their signaling.[109]

3. Prostaglandin-Based Therapeutics/Misoprostol

Neutrophils perform a variety of tasks to protect their hosts from invasion of foreign pathogens. Briefly, and without going into detail, these tasks include cellular adherence, chemotaxis, phagocytosis, degranulation with subsequent enzyme release, production of toxic oxygen metabolites, and inflammatory mediator release. Leukocytes, as mentioned in the first part of this review, are capable of producing various cytokines that have been implicated in inflammation and tissue damage. Elevation of cAMP has been shown to inhibit most of these responses in activated neutrophils as well as inhibit various pro-inflammatory and immune functions in other leukocyte populations.[110-114]

Since inflammatory disease often involves exaggerated or inappropriate responses by leukocytes, drugs that are able to stimulate leukocyte adenylate cyclase might represent potential anti-inflammatory agents. Among those compounds able to raise leukocyte cAMP production, the PGEs have received attention as possible therapeutic agents in immune and non-immune mediated inflammation. The roles of prostanoids in inflammation have been recently described and are beyond the scope of this brief review.[107, 108]

PGE₂, the focus here, is well-known for its inflammatory effects, but it also has anti-inflammatory properties.[107, 108] Signaling through the two Gs-coupled receptors, EP2 and EP4, mediate the dominant aspects of the anti-inflammatory activity of PGE₂.[108] As previously mentioned, these two receptor subtypes signal through increased cAMP.[109]
PGE₂ limits pro-inflammatory cytokine synthesis through EP2 (and EP4) activation.[115, 116] PGE₂ inhibits the production of TNF-α, IL-1β, IL-12, and IL-8 by macrophages.[106] In related studies, PGE₂ has been shown to reduce the production of TNF-α by activated macrophages.[106, 116] In another study, PGE₂ suppressed TNF-α production and enhanced IL-6 production in LPS-stimulated neutrophils.[117] Clinical use of PGE₂ is severely limited by extreme metabolic instability, poor oral absorption, and undesirable side effects.[112] Synthetic analogues of PGES may represent better candidates for dampening immune responses and combating inflammatory disease because of their greater metabolic stability and improved oral absorption.[112] For example, PGE₁ has been shown to elevate cAMP in leukocytes. PGE₁ differs structurally from PGE₂ only by the presence of a double bond at the C-5,6 position of PGE₂.[118] Small structural differences may elicit different clinical effects.

3.1 Potential role for misoprostol: beyond the gastrointestinal tract

Misoprostol is a synthetic analogue of PGE₁ that is relatively stable and orally absorbable in horses.[119] All four PGE receptors are bound by PGE₂. In contrast, misoprostol binds only to EP2, 3, and 4 and is said to be EP2/EP3-selective.[120-122] In humans, the drug is licensed to prevent non-steroidal anti-inflammatory drug-associated peptic ulcers.[119] Misoprostol’s mode of action includes protecting mucosa.[119, 121] The drug has been used clinically in horses to protect against gastrointestinal ulceration induced by NSAID therapy. Misoprostol is also known to have uterogenic activity (including softening of the cervix) and has been used in combination therapy to induce medical
abortions in humans and animals. [119, 121, 123-125] Misoprostol has been reported to have some immunosuppressive and anti-inflammatory effects both in vitro and in vivo, but there are no reports to the author’s knowledge on its clinical use for immunosuppressive and anti-inflammatory purposes in horses.

In human leukocytes, natural PGE₁ and misoprostol have been shown to increase cAMP production in a concentration-dependent manner.[110, 112, 119] Support for a role of misoprostol in preventing consequences of sepsis comes from work in which LPS-induced gastric mucosal damage in study animals (rats and cats) was prevented by treatment with misoprostol. The effects were attributed to inhibition of TNF release.[126, 127] In a related report, misoprostol was shown to inhibit systemic LPS-induced TNF-α levels in vivo.[118] A further study involving human peripheral blood monocytes, showed that misoprostol depressed the LPS-stimulated production of IL-1 and TNF-α. [128][129] Haynes et al. also found that misoprostol regulated inflammatory cytokines from activated human peripheral blood mononuclear cells (PBMCs) and concluded that its actions were mediated through increased cAMP. In this study, misoprostol was shown to inhibit TNF-α and IL-1 release and stimulate IL-6 release.[130] Collectively, these studies suggest a role for misoprostol in preventing the triggering of inflammation. Numerous studies have shown an additive, or possibly synergistic, effect of misoprostol with non-steroidal anti-inflammatory drugs (NSAIDs) in modulating inflammation.[119] For a more in-depth discussion of the immunomodulatory actions of misoprostol and its potential therapeutic applications, the reader is referred to other reviews.[119,121]
4. Conclusion

This section has broadly covered the potential immunomodulatory activities of misoprostol that may suggest a role for it as an adjunctive treatment in SIRS/sepsis. The research presented in this thesis is a first step in the investigation of misoprostol’s potential use in horses with SIRS/sepsis.

C. Priming effects of GM-CSF on leukocyte inflammatory cytokine production

1. Introduction to “priming”

The pluripotent hematopoietic growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) has been extensively studied for its role in differentiation and proliferation of monocytes and granulocytes.[131] Besides these well-documented roles of GM-CSF, the growth factor also potentiates the release of various pro-inflammatory cytokines, including TNF-α and IL-1β, after LPS stimulation without inducing production of these mediators on its own. This amplifying effect on the cellular response is referred to as “priming”. [131] The underlying signaling cascades are not completely elucidated, but recent studies have gained better insight into the cellular mechanisms of priming by GM-CSF.[131]

2. Effects of GM-CSF on LPS-induced TNF-α secretion – specific experimental studies

Lendemans et al. investigated the effects of GM-CSF on LPS-induced TNF-α production. They determined optimal time and dose of GM-CSF priming of LPS-induced TNF-α production in human monocytes. To summarize their experimental protocol, human
peripheral blood mononuclear cells (PBMCs) were pre-incubated with different concentrations of GM-CSF and for different periods of time. After the pre-incubation period, GM-CSF was removed from the medium and cells were stimulated with 10 ng/mL LPS for 4 h. They observed a concentration-dependent increase in LPS-induced TNF-α production that reached its maximum after a period of 6 h and remained stable for 24 h. The maximal response was achieved with a GM-CSF concentration of 100 ng/mL. Under these conditions they found that GM-CSF increased LPS-induced TNF-α production by a factor of 3.4.[131] They did not observe an increase in TNF-α after pre-incubation with GM-CSF in the absence of LPS. The authors also found that both TLR2 and TLR4 expression on human monocytes was up-regulated with GM-CSF, although only a minor portion of the priming response can be attributed to the higher numbers of these LPS receptors.[131] They also examined the signaling cascades involved in GM-CSF priming of LPS-induced TNF-α production. They determined that the enhanced TNF-α synthesis in monocytes pre-incubated in GM-CSF (“primed”) could be attributed to increased phosphorylation of IκB, increased levels of NFκB and AP-1 binding.[131] Complicating this finding, there is no evidence that GM-CSF by itself is able to activate NFκB or induce TNF-α production. They concluded that the signaling cascade comprising of Raf-1, MEK1/2, and ERK1/2 may be involved in GM-CSF-induced priming of TNF-α production.[131] Details of these pathways will not be reviewed here.

In another study investigating the effects of GM-CSF priming on human neutrophils, priming of LPS-induced TNF-α production was achieved at a concentration of 100 ng/mL GM-CSF with a pre-incubation period of 30 min.[132]
4. Conclusion

Collectively, these studies demonstrate the usefulness for GM-CSF priming to induce leukocyte functions, specifically LPS-induced TNF-α synthesis, and provide some insight into the potential signaling pathways involved.
REFERENCES


CHAPTER 2

Effects of the Prostaglandin Analogue Misoprostol on TNF-alpha Release by Activated Equine Leukocytes

ABSTRACT

To investigate the ability of the prostaglandin E1 analogue, misoprostol, to suppress the release of TNF-α from lipopolysaccharide (LPS)-stimulated equine leukocytes, misoprostol was added to equine peripheral blood leukocytes at concentrations of 0.001 μM – 100 μM. An hour later, LPS (E. coli 055:B1) was added at a final concentration of 100 ng/mL to primed leukocytes. Leukocytes were incubated up to 18 h at 37°C, then supernatants were harvested and assayed for tumor necrosis factor-alpha (TNF-α) concentration (ELISA). To investigate the effects of misoprostol in ongoing inflammation, misoprostol was added to cells already stimulated with LPS.

Pre-incubation of LPS-stimulated leukocytes with misoprostol caused significant (p < 0.05) reduction in secretion of TNF-α in a concentration-dependent manner. At concentrations of misoprostol > 0.5 μM, TNF-α production in the supernatant was inhibited significantly, with maximal inhibition at 100 μM. This ability of misoprostol to suppress TNF-α production was maintained in cells incubated in LPS for 30 minutes prior to treatment with misoprostol. When misoprostol treatment was applied 2 h after LPS-stimulation the peak TNF-α secretion was blunted, but values were not statistically different than LPS-treated cells that did not receive misoprostol treatment.

Stimulation with LPS induced TNF-α secretion by equine peripheral blood leukocytes. This effect was blunted by treatment of cells with misoprostol. Misoprostol may
be useful as an immunomodulator of inflammatory cytokine production in systemic inflammatory responses in horses induced by endotoxin. Further \textit{in vivo} and \textit{ex vivo} investigation is warranted.

1. \textbf{INTRODUCTION}

Endotoxin (bacterial lipopolysaccharide; LPS) derived from the outer-membrane of Gram-negative bacteria is accepted as a major causative agent in the pathogenesis of sepsis.\cite{1} The magnitude of LPS-induced equine health problems cannot be over-estimated since endotoxemia is intimately involved in the pathogenesis of gastrointestinal disorders that cause colic, the leading cause of death in adult horses, and bacterial septicemia, the major killer of foals less than 7 days of age.\cite{2} These diseases lead to huge economic losses to the horse industry. The morbidity and mortality associated with endotoxemia and septic shock in horses are primary attributable to the endogenous mediators released during the host’s exaggerated inflammatory response triggered by bacterial LPS.\cite{1,3} Antimicrobials do not mitigate the effects of endotoxin and many current pharmacological therapies, such as non-steroidal inflammatory drugs (NSAIDs), only affect part of the inflammatory cascade; making this a difficult condition to manage effectively. The optimal therapy may involve methods to alter the generation of inflammatory mediators.

Once endotoxins gain access to the blood, immune cells become activated by LPS and release inflammatory mediators, including prostaglandins (PGs) and pro-inflammatory cytokines. Of these mediators, tumor necrosis factor alpha (TNF-\textit{\alpha}) is the principle mediator initiating a cascade of networking inflammatory mediators that results in massive
inflammation, which in turn, is responsible for most of the pathophysiologic consequences of endotoxemia. If this exaggerated immune response is unchecked, it can ultimately lead to multiple organ dysfunction and death.[1]

Therapy directed towards inhibiting PG production largely depends on NSAIDs or corticosteroids. NSAIDs may be effective at relieving some symptoms of inflammation, but may not reduce the long-term progression of disease sustained or mediated by inflammatory cytokines.[4] Corticosteroids are associated with a number of deleterious side effects in horses such as increased incidence and severity of secondary infections and predisposition to laminitis.[5] There is recent interest into the development of therapeutic strategies directed at suppressing the production of inflammatory mediators with less severe side-effects. Such targets for drug therapy include TNF-α. Attempts to neutralize circulating TNF-α through the use of anti-TNF-α antibodies in horses with endotoxemia have not proven to be highly effective, except when administered prior to exposure to LPS.[6,7] Nevertheless, the capacity to neutralize TNF-α production remains a critical goal for investigating novel drugs for use in diseases associated with systemic inflammation.

Misoprostol, a synthetic analogue of PGE₁, has been shown to depress LPS stimulation of TNF-α by activated human monocytes due to its ability to raise leukocyte cyclic adenosine monophosphate (cAMP) production, which has been shown to inhibit release of various pro-inflammatory cytokines by leukocytes.[8,9] In horses, misoprostol has been used clinically as an anti-ulcer drug to treat gastric injury and colon injury caused by NSAIDs.[7] The drug clenbuterol (a β₂-agonist), whose mechanism of action also leads to an increase in the anti-inflammatory intracellular signaling molecule cAMP, is recognized to have anti-
inflammatory effects in horses challenged with a low dose of endotoxin.[4] We propose that there is a potential immunomodulatory role for misoprostol in the therapeutic treatment of systemic inflammatory diseases and sepsis in horses.

Given the capacity of misoprostol to inhibit cytokine release from immune cells in other species and its current clinical use in horses for protection against NSAID-induced gastrointestinal ulcers, the objective of the study reported here was to evaluate the effects of misoprostol on production of TNF-α by LPS-stimulated equine leukocytes. The hypothesis of this study was that misoprostol would suppress LPS-induced secretion of TNF-α by equine peripheral blood leukocytes.

2. MATERIALS AND METHODS

2.1 Horses

Nine adult horses (8 mares and 1 gelding) were randomly selected from the College of Veterinary Medicine Teaching Animal Unit herd and used for the study. Breeds represented included Thoroughbred, Appendix, and Quarter Horse. All horses were healthy, had no recent history of illness or treatments, were in good body condition (BCS 5/9) and were on pasture supplemented with hay/grain. The use of horses in this study was approved by the North Carolina State University Institutional Animal Care and Use Committee.
2.2 Sample collection

Peripheral blood samples were collected via jugular venipuncture into syringes containing heparin. After collection, heparinized whole blood was transferred into sterile endotoxin-free 15 mL conical tubes and red blood cells were allowed to settle for 45 min to 1 h at room temperature. The leukocyte-rich plasma was collected following sedimentation and placed into a sterile endotoxin-free 50 mL conical tube. Cell suspensions were diluted in RPMI 1640 medium as described below.

2.3 Preparation of LPS, GM-CSF, and misoprostol

Lipopolysaccharides (Escherichia coli 055:B5, Sigma, St. Louis, MO) were reconstituted in phosphate buffered saline (PBS) to achieve a concentration of 1 mg/mL. Prior to use, LPS was further diluted into PBS. Equine recombinant GM-CSF (Kingfisher Biotech, St. Paul, MN) was reconstituted in 1% bovine serum albumin (BSA) to achieve a final concentration of 50 μg/mL and further diluted into 1% BSA for experimental use. Misoprostol (Cayman Chemical, Ann Arbor, MI) was supplied as a solution in methyl acetate. The solvent was evaporated under a gentle stream of nitrogen and DMSO (Sigma, St. Louis, MO) was immediately added to achieve a concentration of 200 mM. Prior to use, Misoprostol was reconstituted in RPMI 1640 (Corning Life Sciences, Mediatech Inc., Manassas, VA), to achieve final concentrations of 0.001 μM – 100 μM. All experiments were conducted in triplicate for each horse.
2.4 Stimulation of leukocytes

2.4.1 Pretreatment with misoprostol

Six horses were used for this experiment. Aliquots of leukocyte-rich plasma were pipetted into sterile 1.5-mL microcentrifuge tubes. Cells were rested for 10 minutes, then diluted 1:2 with RPMI 1640 media alone or with either misoprostol (final concentration 0.001, 0.01, 0.1, 0.5, 1, 10, 50, 70, and 100 μM) or equal volume vehicle control (DMSO) and incubated in a 37°C water bath for 1 h. After the incubation period, cells were resuspended by gently flicking the tube. Aliquots of leukocyte cell suspensions were pipetted in triplicate into the wells of sterile flat-bottom 96-well tissue culture plates (200 μl/well) and stimulated with 10 μl LPS at a final concentration of 100 ng/ml or equal volume of phosphate buffered saline (PBS; control). Thirty minutes before LPS stimulation (during the 1 h pretreatment period with misoprostol) the cells were primed with 100 ng/mL GM-CSF. The concentration of LPS (E. coli 055:B5) and GM-CSF were selected based on previously published work and preliminary experiments done by the author (see supplemental methods/results; Fig. S1, S2a and S2b).[11-15] The cells were incubated at 37°C with 5% carbon dioxide (CO₂) for 6 h, and then plates were centrifuged at 250 xg for 5 min, after which the supernatant was aspirated from each well, placed in polypropylene tubes and frozen at -80°C until assayed for TNF-α concentration, as described below. Leukocyte viability was assessed prior to collecting the supernatant using the principle of trypan blue exclusion. Briefly, the cells from each well were stained with 0.4% trypan blue and the number of dye-excluding (live) cells and positively staining (dead) cells were counted using
a hemocytometer. The viability assays were performed in duplicate and results reported as the percentage of viable cells.

2.4.2 **Misoprostol treatment after LPS stimulation**

To determine whether misoprostol could suppress TNF-α production in cells already activated by LPS, equine leukocytes were treated with misoprostol after stimulation with LPS. One hundred microliter aliquots of leukocyte-rich plasma were added to wells of flat-bottom sterile 96-well tissue culture plates and diluted with an equal volume of RPMI 1640. Cells were rested on the plates for 10 minutes, and then were primed with 100 ng/mL GM-CSF as previously described. After a 30 minute incubation with GM-CSF, 10 μl of LPS (or an equal volume of PBS) was added to the wells and the plates were incubated at 37 °C in 5% CO₂. After 30 minutes or 2 hours, cells were treated with 10 μl misoprostol at a final concentration of 50 μM, 100 μM or equal volume of vehicle (DMSO). Each treatment was added to wells in triplicate. Cell supernatants were collected at 2 h, 4 h, 6 h, and 8 h post-treatment and stored at -80°C until analyzed for production of TNF-α. Seven different horses were used for these experiments.

2.5 **TNF-α assay**

Cell culture supernatants were assayed for TNF-α concentration using a commercially available equine-specific TNF-α ELISA kit (Endogen Equine TNF-α screening set; Thermo Fisher Scientific Inc., Rockford, IL, USA). The assay was performed according to manufacturer’s instructions and modified as previously described and validated.[12, 16, 17]
All plasma samples were diluted at a minimum of 1:3 (baseline and controls) or 1:5 (LPS) with reagent diluent (4% BSA in Dulbecco’s PBS, pH 7.4) to avoid a plasma-matrix effect interfering with the assay. Equine recombinant TNF-α (Thermo Fisher Scientific) was used for the standard curve and prepared in reagent diluents to make a top standard of 2000 pg/mL and a low standard of 15.6 pg/mL. SoftMax® Pro Microplate Data Acquisition and Analysis Software was used to analyze data and for generation of the standard curve (Molecular Devices, Sunnyvale, CA). The lower limit of detection of the assay was determined to be 40 pg/mL and is consistent with other studies using the same ELISA kit.[12] Each sample was assayed in triplicate. Samples with values close to or greater than the top standard were further diluted and re-assayed to ensure they were on the straight part of the (sigmoidal) calibration curve. Absorbance was measured on an ELISA plate reader at 550 nm subtracted from 450 nm.

2.6 Data and Statistical analysis

Statistical analysis was performed using the commercially available Sigma Plot statistical software with one-way repeated measures ANOVA analysis (Systat software Inc, San Jose, CA). The software allowed determination of IC₅₀. Significance was only ascribed to p-values < 0.05. Data values are expressed as means ± SEM. The inhibitory effect of misoprostol on TNF-α production was compared with LPS-only stimulated samples (no misoprostol or DMSO vehicle control). For TNF-α concentrations below the lower limit of detection (defined above), a value of 40 pg/mL was assigned for purposes of statistical analysis.
3. RESULTS

3.1 Pretreatment of equine leukocytes with misoprostol reduced production of LPS-induced TNF-α.

In preliminary experiments, we determined that incubation of equine leukocyte-rich plasma with *E. coli* 055:B5 LPS resulted in a concentration-dependent increase in TNF-α secretion. The lowest effective concentration of LPS to achieve a significant increase in TNF-α levels from baseline was 100 ng/mL (data not shown; see supplemental experiments, Fig. S1a and S1b). Supernatant concentrations of TNF-α were significantly increased by LPS (100 ng/mL) + GM-CSF at 4 h, 6 h, 18 h, and 24 h in comparison to the negative control (PBS [VC]) and Time 0 untreated cells (p < 0.05). GM-CSF (100 ng/mL) pre-treatment effectively primed LPS-induced TNF-α production by leukocytes; however, GM-CSF alone did not significantly induce TNF-α production. LPS alone stimulated TNF-α production by leukocytes, but this response was not significantly different from baseline (data not shown; see supplemental experiments, Fig. S2). Peak production of TNF-α levels in cell supernatants were detected at 4 to 6 h after stimulation, with horse-to-horse variation observed (data not shown; Fig. S2).

TNF-α was detected in supernatants from equine peripheral blood leukocytes in the absence of LPS-stimulation (baseline [time 0] or untreated [PBS control]); however, there was a significant main effect (p < 0.05) of added LPS + GM-CSF on TNF-α production when all the data was considered (Fig. S1a, Fig. S1b, and Fig. S2).
Pre-incubation of equine leukocytes with misoprostol inhibited LPS-induced secretion of TNF-α in a concentration-dependent manner, with a calculated IC\textsubscript{50} value of 1.04 μM (Figs. 1 and 2). Misoprostol significantly blunted TNF-α production at concentrations of 0.5 μM – 100 μM compared to untreated LPS-stimulated control cells (p < 0.5). Maximal inhibition was observed at a concentration of 100 μM; however, there was a trend towards a plateau in inhibition beginning at a concentration of 50 μM (Fig 1). Although there was less TNF-α measured from samples pretreated with DMSO (LPS + VC), the vehicle did not significantly suppress TNF-α production (Fig. 1 and S3).

There was not an effect of LPS or misoprostol treatment on leukocyte viability (> 95% viability at 6 h and > 90% at 18 h) (data not shown), confirming that the effects of misoprostol on cell secretion of TNF-α were not due to a reduction in cell viability.

Collectively, this data indicates that LPS is a potent inducer of TNF-α production from equine leukocytes and peak TNF-α production was inhibited with pretreatment with misoprostol 30 minutes before LPS stimulation.

3.2 Misoprostol maintains its ability to suppress TNF-α production when added after LPS activation.

To investigate whether misoprostol maintains its ability to reduce TNF-α secretion in the face of ongoing inflammation, misoprostol was added at a concentration of 50 μM or 100 μM (based on results of the previous experiment) to leukocyte cultures 30 min or 2 h after LPS stimulation. TNF-α in cellular supernatants of cells pre-incubated in LPS (+ GM-CSF) remained significantly lower in cells treated with both 50 μM and 100 μM concentrations of
misoprostol after 30 min of LPS stimulation compared to untreated stimulated cells (LPS alone) or cells treated with vehicle (LPS + VC) at 4 h, 6 h, and 8 h post-misoprostol treatment (p < 0.05) (Fig. 3). There was not a significant difference in TNF-α production from cells incubated with LPS alone and LPS + misoprostol at 2 h post-misoprostol treatment; however, this is earlier than the expected peak of TNF-α secretion. Leukocytes incubated with medium alone (untreated) did not produce TNF-α above baseline (time 0). A similar observation was made when misoprostol was applied 2 h after LPS stimulation; however, this trend was not statistically significant (Fig. 4) at any time of sampling. Peak TNF-α production by leukocytes was collectively much lower in all LPS-stimulated groups than in the previous experiment (3500 ± 1023 pg/mL vs. 9265 ± 2200 pg/mL), and after an initial increase from baseline (observed at 2 h post-misoprostol treatment), TNF-α production appeared to plateau. Baseline TNF-α (time 0) and TNF-α in untreated cells (medium alone) were also higher in the group of horses treated with misoprostol 30 min after stimulation than in the group treated 2 h after LPS stimulation with a high horse-to-horse variability (baseline: 1882 ± 1515 pg/mL vs. 462 ± 423 pg/mL).

Collectively, these data show that misoprostol maintains its ability to suppress TNF-α production when administered 30 minutes after E. coli 055:B4 LPS activation, but not significantly when administered 2 hours after LPS.
4. DISCUSSION

Sepsis and syndromes associated with an exaggerated inflammatory response remain common clinical entities in human and veterinary species. Administration of antimicrobials (although controversial) in combination with non-steroidal anti-inflammatory drugs (NSAIDs) is the cornerstone of treatment of these conditions in horses, but medications that interfere with the network of inflammatory mediators involved may lessen the inflammatory response associated with sepsis and decrease patient morbidity and mortality.[18] The study presented here was an *in vitro* assessment on the ability of the synthetic prostaglandin E₁ analogue misoprostol to inhibit a key inflammatory cytokine critical in the pathophysiology of sepsis. The results of this study supported the expectation that misoprostol would inhibit TNF-α release by LPS-activated equine peripheral blood leukocytes.

The experimental model of LPS *in vitro* activation of leukocytes used in this study is well-established and previously published by this laboratory.[11, 15] However, in the study reported here, the minimum stimulatory concentration of LPS (*E. coli* 055:B5) significantly increasing TNF-α production from unstimulated controls was 100 ng/mL compared with 10 ng/mL used in related experiments evaluating leukocyte activation by this laboratory (data not shown).[15] Other published studies have induced TNF-α release from peripheral blood mononuclear cells with a concentration of LPS (100 ng/mL) similar to what was used in this study.[9, 12] In the experimental model reported here, cells were primed with recombinant equine GM-CSF prior to stimulation with LPS to mimic responses *in vivo* during initiation of the inflammatory response. “Priming” is broadly defined as the process of amplifying the cellular responses to an activating stimulus by prior exposure to a priming agent.[14] GM-
CSF has a role of a potentiator of the early stage of naturally occurring sepsis because it has a priming effect on immune cells for activation with an inflammatory stimulus such as bacterial endotoxin.[19] The priming effects of GM-CSF on LPS-induced TNF-α synthesis are well-known.[13, 14] The concentration of GM-CSF (100 ng/mL) and pre-incubation period (30 min) with the priming agent used in this study were selected somewhat empirically based on combined results of preliminary work in our laboratory and on research reported by others.[13, 14] The underlying signaling cascades involved in GM-CSF priming of LPS-induced TNF-α production by leukocytes are not completely elucidated, but may be partly due to up-regulation of LPS receptors on the surface of inflammatory cells and involve the signaling cascade comprising of Raf-1, MEK1/2, and ERK1/2.[13] Other evidence suggests involvement of the NF-κB signaling pathway.[13] In accordance with other studies, GM-CSF potentiated LPS-induced TNF-α production without inducing the mediator on its own.[13, 14]

As mentioned, TNF-α is pivotal to initiating the pro-inflammatory cascade of mediators in sepsis and is released mainly from mononuclear cells, but also from polymorphonuclear phagocytes after exposure to endotoxin (LPS). Production of TNF-α requires LPS binding protein (LBP) for binding of LPS to the CD14 surface receptor on inflammatory cells and subsequent activation.[19] LBP is naturally present in serum or plasma.[20] In the experimental model presented here, leukocyte-rich plasma was used to provide a source of LBP, as opposed to common models using isolated peripheral blood mononuclear cells (PBMCs) or monocytes that required medium supplemented with serum.
In the present study, LPS significantly induced the production of TNF-α by leukocytes. Experiments were designed to assess the ability of misoprostol to antagonize this effect of LPS-activation of leukocytes. Analyses of results of the study indicate that misoprostol effectively suppressed TNF-α production by LPS-activated leukocytes in a concentration-dependent manner (with an IC₅₀ value of 1.04 μM and maximal inhibitory concentration of 100 μM) when misoprostol was added 1 h before LPS stimulation. In a related study using LPS-stimulated human monocytes, misoprostol (1μM - 100μM) inhibited TNF-α release with an IC₅₀ value of 75 μM.[8] Significantly lower concentrations of TNF-α in cellular supernatant were measured in the misoprostol treatment groups compared to untreated control groups when treated with a concentration of misoprostol between 0.5 μM and 100 μM. These concentrations are similar to those reported in human in vitro studies.[8, 9] On the basis of encouraging results from this study design, additional experiments were performed in which misoprostol treatment was applied after LPS stimulation in an effort to more closely simulate events in clinical settings. In the study reported here, the increased production of TNF-α that was observed after incubation with LPS was significantly inhibited by the addition of misoprostol to the cultures 30 minutes after stimulation and was partly inhibited by misoprostol treatment 2 h after stimulation (50 μM and 100 μM misoprostol), suggesting that misoprostol may be able to blunt TNF-α production induced by LPS in the face of ongoing inflammation. These results were not unexpected, because in most in vivo studies, treatments designed to reduce circulating TNF-α concentration were most effective when administered prior to an endotoxin challenge.[21] Because LPS-induced production of
TNF-α was blunted by misoprostol treatment, the authors propose that misoprostol may have a role as an adjunctive therapy in the treatment of systemic inflammation and sepsis in horses. Again, in the present study, significant decreases in TNF-α were achieved when leukocytes were pre-treated with misoprostol or exposed to the drug within a short time period following LPS stimulation. It is accepted that this is a different situation to clinical cases of inflammation or sepsis, which may be ongoing for several days. In order to further evaluate this drug as a modulator of systemic inflammation, its effects will need to be evaluated in vivo. It is unknown if misoprostol will significantly decrease pro-inflammatory cytokine levels in horses at clinically relevant doses.

On the basis of the analysis of findings for the study reported here, it was determined that LPS at a concentration of 100 ng/mL and treatment with misoprostol or its vehicle control was not directly cytotoxic; incubation of cells with concentrations of misoprostol ranging from 0.001 μM to 100 μM did not result in a significant loss of viability as determined via trypan blue dye exclusion (data not shown). It should be noted that lower levels of TNF-α were measured in samples treated with vehicle control (DMSO); however, the levels of TNF-α were not significantly different from those from LPS stimulation alone. The misoprostol formulation used in this study contained < 0.01% DMSO which should not be cytotoxic. This suppression of TNF-α observed in cells treated with DMSO alone suggests some inhibitory effect of the vehicle, but the effect was not significant enough to explain the inhibition of TNF-α attributed to misoprostol treatment in this study.

Peak TNF-α levels in cellular supernatants were observed at 4 to 6 h after LPS stimulation. Collectively from several experiments, the average peak TNF-α level in cells
primed with GM-CSF and stimulated with LPS was 6861 ± 1955 pg/mL. This average peak TNF-α level cannot be compared to other studies because no reports have been found in horses using GM-CSF priming of leukocytes for LPS-induced TNF-α synthesis. A recent study by Figueiredo et al. reported a peak in TNF-α protein production in supernatants of monocytes incubated with *E. coli* LPS at 8 h; however, the cell type and culture system varied from the present study and the researchers used a lower concentration of LPS (100 pg/mL) and different strain (*E. coli* 0111:B4) for stimulation.22 TNF-α levels remained significantly higher than baseline at 18-24 h and this may reflect ongoing stimulation in this model. Cell viability was determined to remain high (> 90%; data not shown) at these later times of sampling, suggesting that cell death was likely not contributing to the observed TNF-α production by leukocytes.

Variability in average resting plasma TNF-α level (782.9 ± 580.2 pg/mL) was observed among subjects in the present study which may suggest subclinical inflammation in some study subjects although they appeared to be healthy. This variability was especially apparent in the 3 horses sampled for the experiment when misoprostol treatment was applied to cell suspensions 2 h after LPS stimulation. The data from the 3 individual horses, when averaged, collectively produced less TNF-α in response to LPS stimulation as well as had higher baseline levels of TNF-α than was seen in previous experiments reported in this study. Although the samples were handled similarly, the reagents prepared the same, and the experiment was conducted under the same experimental conditions (with the exception of the time of misoprostol treatment in relation to LPS-stimulation), experimental error as a reason for these ambiguous results cannot be excluded. For example, these results might be
explained if adequate priming of leukocytes was not achieved with GM-CSF. Because of this high horse-to-horse variation in this experiment and lower levels of TNF-α produced by leukocytes in response to LPS stimulation compared to other experiments in this study, the results reported with misoprostol treatment 2 h after exposure to LPS should be interpreted with caution. Also noteworthy is the high S.E.M. for baseline TNF-α secretion (1881.9 ± 1514.9 pg/mL) in the group of horses sampled for the 30 min post-LPS experiment. One individual horse had a very high baseline TNF-α level (6400 pg/mL). This could be attributed to stimulation during the sampling or handling process, but cells showed high response to LPS, decreasing the likelihood of significant stimulation during sample handling. Of note, Lavoie-Lamoureaux et al. report that quantitative differences can be found in recombinant TNF-α standards between lots of ELISA kits from the same manufacturer and this could also have an impact on absolute TNF-α values.[23] ELISA kits with different lot numbers were used for these experiments. Although horse-to-horse variability in response to LPS was large in the experiments reported here, the inhibitory effects of misoprostol on TNF-α were consistent, especially when analyzing the data from the experiments with misoprostol added before LPS and misoprostol added 30 min after LPS stimulation. This variation in response to LPS is consistent with the results of other studies on the effects of LPS in horses.[24, 25] There have been some other noteworthy trends with TNF-α secretion reported in other studies that cannot be ignored as potential reasons for horse-to-horse variation in TNF-α. Interestingly, in obese horses, increased plasma TNF-α concentrations were associated with increased body condition score.[16] None of the horses included in this study, however, were considered by the author to be obese or having physical characteristics
of equine metabolic syndrome. Some of the mares used in the present studies were > 15 years. McFarlane and Holbrook reported that release of TNF-α from unstimulated PBMCs in culture was similar among adult horses, aged horses, and horses with PPID. However, in that study, higher levels of TNF-α were observed from LPS-stimulated PBMCs collected from PPID horses.[12] Although none of the horses in this study had phenotypic characteristics of PPID, the condition could not be excluded without further testing and this may explain some of the horse-to-horse variation in LPS-induced TNF-α production. In contradiction to the findings of similar TNF-α levels among LPS-stimulated PBMCs from adult and aged horses reported by McFarlane and Hobrook, Adams et al. found significant levels of baseline TNF-α in the serum of old horses and attributed it to “inflamm-aging.” It is possible that the horse-to-horse variations reported in the present study are due to age-related differences in TNF-α in the serum reflecting the dysfunctional activity of senescent cells in vivo.[26] There was also horse-to-horse variation in LPS-induced production of TNF-α in the study reported here, suggesting that individual horses respond differently to the LPS stimulus. These marked variabilities could conceivably obscure potential beneficial responses to misoprostol in future in vivo studies.

Certainly, the small number of horses sampled was a limitation of this study. Another limitation of this study was the lack of inclusion of horses across all age ranges as most of the study horses were middle-aged to aged. In addition, the same group of horses were not used for each experiment due to IACUC restrictions in frequent sampling, adding to the variability in baseline and peak TNF-α observed across experiments. It should also be noted that the author found that factors in the plasma appeared to interfere with the ELISA assay,
underestimating the TNF-α concentrations when the assay was performed per manufacturer’s directions. The effects of these factors were minimized by diluting the samples at a minimum of a 1:3 dilution in reagent diluent, as has been previously described by Vick et al. and confirmed in our laboratory.[16]

We were interested in investigating misoprostol for treatment of horses with systemic inflammation because misoprostol has been reported to have some immunosuppressive and anti-inflammatory effects both in vitro and in vivo in other species. There are no reports to the author’s knowledge on its clinical use for immunosuppressive and anti-inflammatory purposes in horses. Support for a role of misoprostol in preventing consequences of sepsis comes from work in which LPS-induced gastric mucosal damage in a model of septic shock in rats and cats was prevented by treatment with misoprostol. The effects were attributed to inhibition of TNF-α release.[27, 28] In a related report, misoprostol was shown to inhibit systemic LPS-induced TNF-α levels in vivo.[29] A number of studies involving human peripheral blood leukocytes, showed that misoprostol depressed the LPS-stimulated production of TNF-α as well as IL-1, another important pro-inflammatory cytokine implicated in the pathophysiology of systemic inflammation and sepsis.[8,9, 30] Misoprostol was also found to stimulate IL-6 release, a comparatively less acutely cytotoxic cytokine and important stimulator of hepatic acute phase proteins.[30] A function of these acute phase proteins is to help restore homeostasis in inflamed tissue; therefore, increased IL-6 may lead to an overall reduction of inflammation.[30]

In humans, misoprostol is licensed to prevent NSAID-associated peptic ulcers and has been used empirically in horses to protect against NSAID-induced gastric ulcers and right
dorsal colitis due to its mucosal protective properties.[10] Numerous studies have shown an additive, or possibly synergistic, effect of misoprostol with NSAIDs in modulating inflammation.[31] NSAIDs act by inhibiting the activity of cyclooxygenase (COX) enzymes, whereas the synthetic prostaglandin E₁ analogue misoprostol exerts its anti-inflammatory effects by signaling through the G-protein-coupled E-prostanol receptors EP2 (and possibly EP4) that are present on many cell types of the immune system and signal through increased cAMP.[10] Misoprostol is selective for the EP2 and EP3 receptors (the latter is a low affinity receptor) and is relatively stable and orally absorbable.[10] This is in contrast to naturally occurring PGE2, which use is severely limited clinically by extreme metabolic instability, poor oral absorption and undesirable side effects.[32]

Other classes of drugs such as the phosphodiesterase inhibitor pentoxifylline and the β-agonist drug clenbuterol will also increase cAMP levels within leukocytes. Benefits of using pentoxifylline in in vivo models of sepsis have been equivocal.[33] Cudmore et al. reported measurable anti-inflammatory effects of clenbuterol in horses challenged with a low dose of endotoxin.[4] Combination therapy of NSAIDs with drugs that act to increase cAMP levels in leukocytes, and ultimately suppress TNF-α production, may provide superior anti-inflammatory effects when compared to NSAIDs alone.

Although many in vitro studies have suggested that treatment with anti-TNF-α antibodies may be beneficial, this has not translated from benchtop to patient-side and results have been disappointing.[34] In vivo studies have revealed no improvement, or even detrimental effects, of anti-TNF-α therapy. Apparent failure of these anti-TNF-α antibody treatments may be due to increased concentrations of cell-associated TNF-α despite
effectively reducing secreted amounts of bioactive TNF-α.[35] A recent study suggests that inhibition of de novo synthesis of TNF-α is likely to be more effective than anti-TNF-α drugs.[35] Furthermore, in clinical studies in humans, many of the patients were frequently in a state of immunoparalysis and mortality associated with sepsis could not be explained by an uncontrollable “cytokine storm”.[36, 37] In equine patients, the balance between pro-inflammatory and anti-inflammatory mediators may more frequently culminate in systemic inflammation vs. immunosuppression.[38] However, it is possible that any treatment predominately targeting TNF-α suppression may have limited clinical use in horses. It should be recognized that the concentration of misoprostol used in this study may not correlate to safe dosages for clinical use. Misoprostol is associated with unpleasant side effects such as abdominal cramping and diarrhea; therefore, these severe side effects may preclude the administration of effective inflammatory doses.

Future directions include evaluating the immunomodulating effect of misoprostol on production of other important cytokines involved in the systemic inflammatory response, such as the pro-inflammatory mediators IL-1β, IL-6, IL-8 and interferon-γ; as well as the anti-inflammatory mediator IL-10. Investigation into the ex vivo and in vivo effects of misoprostol in endotoxin-challenged horses is also warranted.

5. CONCLUSIONS

TNF-α plays a pivotal role in systemic inflammatory response syndromes (SIRS) and sepsis; therefore, reducing its activity would be expected to have beneficial effects on outcome. Based on this preliminary in vitro study, misoprostol may hold promise as an
adjunctive therapy combined with conventional treatments in SIRS and sepsis conditions in horses. Because of the early role of TNF-α in sepsis, because TNF-α is only one of several inflammatory mediators involved in sepsis, and because not all patients have increased levels of circulating TNF-α, it is unlikely that monotherapy targeting TNF production will prove to be beneficial in all patients with clinical sepsis. Until further studies evaluate the in vivo effects of misoprostol on TNF-α and other inflammatory mediator production, this drug cannot be currently recommended as a therapy.

6. CONFLICT OF INTEREST STATEMENT

None declared.

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REFERENCES


Figure 1: Effects of misoprostol on LPS-induced TNF-α production by equine peripheral blood leukocytes. Values represent the mean ± S.E.M. for 6 horses. * Significantly different from value for LPS alone. + Significantly different value from LPS+VC (p < 0.05)
Figure 2: Effects of misoprostol on LPS-induced TNF-α production by equine peripheral blood leukocytes. IC_{50} = 1.04 μM
Figure 3: Effects of 50 μM and 100 μM misoprostol on LPS-induced TNF-α production when treated with misoprostol 30 minutes after LPS stimulation. Values represent the mean ± S.E.M. for 4 horses. * Significant difference between misoprostol treated samples (LPS + M 50 and LPS + M 100) and LPS alone. + Significant difference between misoprostol treated samples and LPS + VC. (p < 0.05)
Figure 4: Effects of 50 μM and 100 μM misoprostol on LPS-induced TNF-α production when treated with misoprostol 2 h after LPS stimulation. Values represent the mean ± S.E.M. for 3 horses. Treatment was not statistically different from LPS alone or LPS+VC.
Appendix A

APPENDICES

SUPPLEMENTAL METHODS

S1. LPS stimulates TNF-α production from equine leukocytes.

Leukocyte-rich plasma was aliquoted into the wells of 96-well tissue culture plates (200 µl/well). After resting on the plates for 10 min, the cells were primed with 100 ng/mL GM-CSF for 30 min, then stimulated with LPS (at a concentration of either 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 100 ng/ml or 1000 ng/ml or equal volume of vehicle control (PBS). The cells were incubated at 37°C in 5% carbon dioxide (CO2) for 6 h or 18 h; then plates were centrifuged at 250 xg for 5 min after which the supernatant was collected, placed in polypropylene tubes and frozen at -80°C until assayed for TNF-α concentration.

S2. GM-CSF treatment of neutrophils.

To determine whether GM-CSF priming would enhance LPS-stimulated cytokine production from equine leukocytes, aliquots of leukocyte-rich plasma were pipetted into the wells of sterile 96-well flat bottom plates and cultured in the presence or absence of 100 ng/mL recombinant equine granulocyte macrophage colony stimulating factor (GM-CSF) for 30 min. Cells were then stimulated with 100 ng/mL LPS (or PBS vehicle control) for 24 h. The concentrations of GM-CSF and LPS were selected based on previously published
work.[11-15] Leukocyte viability was determined by trypan dye exclusion; then cell supernatants were harvested at 2 h, 4 h, 6 h, 18 h, and 24 h and stored at -80°C until assayed for TNF-α.

**S3. Misoprostol inhibits TNF-α production in LPS-stimulated equine leukocytes while its vehicle control (DMSO) does not.**

To determine if the vehicle DMSO affected the observed misoprostol suppression of TNF-α production, equine leukocytes were pre-incubated for 1 hour with various concentrations of misoprostol (0.001 μM – 100 μM) or their respective concentration of DMSO vehicle, and then stimulated with 100 ng/mL LPS as previously described. In all groups, cells were primed with 100 ng/mL GM-CSF 30 minutes prior to LPS stimulation. Cell supernatants were harvested at 6 h and stored at -80°C until assayed for TNF-α.
SUPPLEMENTAL RESULTS

S1. LPS stimulates TNF-α production from equine leukocytes

To determine a suitable concentration of LPS to use in the rest of the study, supernatant concentrations of TNF-α were measured after leukocytes were incubated in a wide range of concentrations of LPS. Exposure of equine peripheral blood leukocytes to LPS resulted in a concentration-dependent increase in TNF-α production. An increase in TNF-α levels from baseline and the untreated control group was observed in cell supernatants at all concentrations of LPS (0.1 ng/mL – 1000 ng/mL) at 6 h and 18 h, with statistical significance at concentrations of 100 ng/mL and 1000 ng/mL LPS (p < 0.05). The lowest concentration tested that induced maximal TNF-α was 100 ng/mL (Figs. S1a and S1b). This concentration of LPS was used in subsequent experiments.

S2. GM-CSF priming enhances LPS-stimulated TNF-α production from leukocytes

Exposure of equine peripheral blood leukocytes to LPS resulted in a time-dependent increase in TNF-α production. GM-CSF priming enhanced LPS-stimulated TNF-α production. Elevated TNF-α levels from baseline were detected from both primed and unprimed stimulated cells from 2 h to 24 h. TNF-α production peaked at 4 h post-stimulation, with the highest TNF-α detected from LPS-stimulated leukocytes primed with GM-CSF (Fig. S2). There was a statistically significant difference in TNF-α production from
cells primed with GM-CSF compared to unprimed cells at 4 h, 6 h, 18 h, and 24 h post-LPS stimulation. The peak TNF-α was $11,179.4 \pm 3047.7$ pg/mL for cells primed with GM-CSF compared to $5678.5 \pm 2808.1$ pg/mL for cells stimulated with LPS alone. The peak plasma TNF-α for one horse was at 6 h, suggesting individual horse variability and sensitivity to LPS (individual data not shown). GM-CSF alone did not stimulate TNF-α production. Incubation of cells up to 18 h did not result in a significant loss of viability as determined by trypan dye exclusion. In contrast, cells incubated up to 24 h showed a higher percentage of non-viable cells. This may explain the higher TNF-α level at 24 h compared to 18 h, since TNF-α may be released upon cell death.

S3. Misoprostol inhibits TNF-α production in LPS-stimulated equine leukocytes while its vehicle control (DMSO) does not.

Misoprostol inhibited TNF-α production from LPS-stimulated equine leukocytes in a concentration-dependent manner (Fig. S3). Although TNF-α production by cells treated with LPS + VC was suppressed from LPS alone, a plateau was observed in the initial suppression that was well-above baseline (data not shown) and the corresponding concentration of misoprostol. This data suggests that the inhibition of TNF-α production by misoprostol cannot be explained by the concentration of its vehicle DMSO. The sample size was too small to determine significance; although other findings presented in another portion of this work comparing 100 μM of misoprostol and its corresponding concentration of vehicle control, show that inhibition of TNF- by misoprostol was significantly different from LPS-stimulated cells treated with vehicle control.
Figure S1a: Effects of LPS on TNF-α production by equine peripheral blood leukocytes. Cells were pre-incubated with or without 100 ng/mL GM-CSF and stimulated with LPS (0.1 – 1000 ng/mL) or vehicle control (untreated) for 6 h. “Baseline” represents time 0. Values represent the mean ± S.E.M. for 3 horses. * Significantly different from value for baseline and untreated samples. (p < 0.05)
**Figure S1b:** Effects of LPS on TNF-α production by equine peripheral blood leukocytes.

Cells were pre-incubated with or without 100 ng/mL GM-CSF and stimulated with LPS (0.1 - 1000 ng/mL) or vehicle control (untreated) for 18 h. “Baseline” represents time 0. Values represent the mean ± S.E.M. for 3 horses. *Significantly different from value for baseline and untreated samples. (p < 0.05)
Figure S2: Effects of GM-CSF priming on LPS-induced TNF-α production by equine peripheral blood leukocytes. Cells were preincubated with GM-CSF at a concentration of 100 ng/mL for 30 minutes before stimulation with 100 ng/mL LPS or equal volume of vehicle control for 2 h, 4 h, 6 h, 18 h, and 24 h. “Baseline” represents time 0. Values represent mean ± S.E.M. for 3 horses. * Significant difference between LPS + GM-CSF and LPS alone. (p < 0.05)
Figure S3: Treatment with vehicle control does not have an effect on LPS-induced TNF-α secretion compared to misoprostol. Data is from 2 horses. Statistical significance was not determined.